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Pharmacologic effects of cannabidiol on acute reperfused myocardial infarction in rabbits: evaluated with 3.0T cardiac magnetic resonance imaging and histopathology

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Corresponding Author:	Yicheng Ni, M.D., Ph.D. K.U.Leuven Leuven, BELGIUM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	K.U.Leuven
Corresponding Author's Secondary Institution:	
First Author:	Yuanbo Feng, MD., PhD.
First Author Secondary Information:	
Order of Authors:	Yuanbo Feng, MD., PhD.
	Feng Chen, MD., PhD.
	Ting Yin, MSc.
	Qian Xia, MD., PhD.
	Liu Yewei, MSc.
	Huang Gang, MD., PhD.
	Jian Zhang, PhD.
	Raymond Oyen, MD., PhD.
	Yicheng Ni, M.D., Ph.D.
Order of Authors Secondary Information:	
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Abstract:	Cannabidiol (CBD) has anti-inflammatory effects. We explored its therapeutic effects on cardiac ischemia-reperfusion injury with an experimental imaging platform. Reperfused acute myocardial infarction (AMI) was induced in rabbits with a 90-min coronary artery occlusion followed by 24-h reperfusion. Before reperfusion, rabbits received two intravenous doses of 100 μg/kg CBD (n=10) or vehicle (control, n=10). Evans blue was intravenously injected for later detection of the AMI-core. Cardiac magnetic resonance imaging (cMRI) was performed to evaluate cardiac morphology and function. After euthanasia, blood Troponin I (cTnI) was assessed, and the heart was excised and infused with multifunctional red-iodized-oil dye. The heart was sliced for digital radiography to quantify the perfusion density rate (PDR), area at risk (AAR), and myocardial salvage index (MSI), followed by histomorphologic staining. Compared to controls, CBD treatment improved systolic wall thickening ($p<0.05$), significantly increased blood flow in the AAR ($p<0.05$), significantly decreased microvascular obstruction ($p<0.05$), increased the PDR by 1.7-fold, lowered the AMI-core/AAR ratio ($p<0.05$), and increased the MSI ($p<0.05$). These improvements were associated with reductions in serum cTnI, cardiac leukocyte infiltration, and myocellular apoptosis ($p<0.05$). Thus, CBD therapy reduced AMI size and facilitated restoration of LV

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Suggested Reviewers:	Huaijun Wang, MD, PhD Stanford Hospital and Clinics whjwang@stanford.edu
	Xiaoming Yang, MD, PhD University of Washington School of Medicine xmyang@u.washington.edu
	Robert Dondelinger , MD, PhD Department of Medical Imaging, University Hospital of Liege rdondelinger@ulg.ac.be
Opposed Reviewers:	

UZ LEUVEN | RADIOLOGIE

March 25, 2015

Dear Dr. Michael R. Rosen, Editor-in-Chief of ***journal of Cardiovascular Pharmacology,***

According your suggestions, our manuscript titled "**Pharmacologic effects of cannabidiol on acute reperfused myocardial infarction in rabbits: evaluated with 3.0T cardiac MRI and histopathology"** has been edited by a professional editing service company in USA. We hereby resubmit this manuscript for your consideration of its possible publication in journal of cardiovascular pharmocology.

This manuscript describes in vivo and postmortem evaluation of the therapeutic effects of cannabidiol (the constituent of the plant Cannabis sativa) on ischemic heart disease in a rabbit model. A few key components of acute myocardial infarction have been experimented and analyzed by using a 3.0T clinical MRI scanner and a novel custom-made multifunctional staining. We believe that the demonstrated research platform, applicable methodology and the promising results may substantially help the translational cardiology research and eventually contribute to the ever-enhancing impact factors of your journal.

We declare that all the authors have agreed on the submission and have participated in the study to a sufficient extent to be entitled as co-authors. We also declare no conflicts of interest in the study and full compliance of the study to the Ethical Adherence.

We appreciate in advance your attention and favorable consideration.

Best regards,

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Yicheng NI, MD, PhD
Full professor, Bayer Lecture Chair,
Head of Theragnostic Laboratory,
Department of Imaging and Pathology
Faculty of Medicine, KU Leuven
Herestraat 49, B-3000, Leuven, Belgium
Tel: 0032-16-330165; Fax: 0032-16-343765
e-mail: yicheng.ni@med.kuleuven.be
website: http://www.kuleuven.be/cv/u0014498e.htm
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Pharmacologic effects of cannabidiol on acute reperfused myocardial infarction in rabbits: evaluated with 3.0T cardiac magnetic resonance imaging and histopathology

Running title: 3.0T cardiac MRI for AMI therapy assessment

Yuanbo Feng MD, PhD[1,4]; Feng Chen MD, PhD[1,2];Yin Ting MSc[1]; Qian Xia MD, PhD[1,3]; Yewei Liu MSc[1,3]; Gang Huang MD, PhD[3]; Jian Zhang PhD[4]; Raymond Oyen MD, PhD[1]; Yicheng Ni MD, PhD[1*]

[1]KU Leuven, Department of Imaging and Pathology, University Hospital Gasthuisberg, Leuven, Belgium
[2]Department of Radiology, the First Affiliated Hospital, Zhejiang University, China
[3]Department of Nuclear Medicine, Ren Ji Hospital, Medical School of Shanghai Jiao Tong University, China
[4]Laboratory of Translational Medicine, Jiangsu Academy of Traditional Chinese Medicine, Nanjing, China

*Corresponding author

Prof. Dr. Yicheng Ni MD, PhD, holds Bayer Lecture Chair.

Radiology, University Hospitals, KU Leuven

Herestraat 49, 3000 Leuven, Belgium

Email: yicheng.ni@med.kuleuven.be

Tel: 0032-16-330165; Fax: 0032-16-343765

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Conflict of interest

Authors report no conflicts of interest.

Reprints: Prof. Dr. Yicheng Ni MD, PhD, Radiology, University Hospitals, KU Leuven

Herestraat 49, 3000 Leuven, Belgium. Email: yicheng.ni@med.kuleuven.be

Tel: 0032-16-330165; Fax: 0032-16-343765

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Key words: Rabbit, myocardial infarction, cMRI, cannabidiol, multifunctional staining

Introduction

Ischemic heart disease is a major cause of mortality and heart failure. Extensive research on pharmacological agents has provided therapeutic and interventional solutions.[1] Moreover, advances in imaging technology have played an increasingly important role in translational medicine for developing new diagnostics and therapeutics.

Cannabinoids are the constituents of *Cannabis sativa,* the plant known as marijuana [2]. Most previous studies on cannabinoids have focused on effects in the central nervous system, including the activation of multiple cannabinoid receptors, notably cannabinoid-1 and -2 (CB1 and CB2) receptors [3, 4]. Nevertheless, a growing body of evidence has indicated that cannabinoids are also associated with beneficial effects on physiopathologies in the cardiovascular system [5, 6]. For example, cannabinoids were found to play a protective role in atherosclerosis and myocardial ischemia [2, 7].

Cannabidiol (CBD) is one of the most abundant cannabinoids (Figure 1A). In contrast to the major cannabinoid, Δ9-tetrahydrocannabinol, CBD binds very weakly to CB1 and CB2 receptors, and it does not induce psychoactive or cognitive effects [5, 8]. However, CBD has exhibited multiple pharmacological actions; it has exhibited anxiolytic, antipsychotic, antiemetic, antioxidant, anti-inflammatory, and immunomodulatory properties [8-12]. Moreover, CBD is well tolerated and has no known side effects when chronically administered to humans [9, 10]; thus, it can potentially be safely incorporated into clinical practice. There is also evidence

that CBD has positive effects on the cardiovascular system [13]. When applied directly to isolated arteries, CBD caused both acute and time-dependent vasorelaxation [14]. CBD protected against vascular damage by attenuating oxidative/nitrative stress, inflammation, cell death, and fibrosis in the high glucose environment of a rat model of type 2 diabetes [15]. Recently, acute or chronic CBD treatment was shown to have little effect on hemodynamics and ischemic cardiac arrhythmias, but it protected against ischemia-reperfusion damage, and it reduced the infarct size in rats [2, 7]. CBD influenced the survival, migration, and death of white blood cells, and it enhanced platelet aggregation [13]. Although the precise pharmacological effects of CBD remain to be fully elucidated, all these preclinical data appeared to support the notion that CBD could be beneficial for treating ischemic heart disease.

To date, the majority of CBD studies have focused on small rodent models, and CBD has not been investigated in medium-sized or large animal models. Few articles have addressed CBD therapeutic effects on global and regional cardiac functions and on blood biomarkers specific for cardiac damage, like troponin I (cTnI). Furthermore, no study has evaluated how CBD might affect other important aspects of heart disease, such as the myocardial area at risk (AAR), the myocardial salvage index (MSI), and the myocardial no-reflow phenomenon after treatment. Thus, to investigate the role of CBD in ischemic heart disease, it may be necessary to work in other animal models. Cardiac magnetic resonance imaging (cMRI) is a widely applied noninvasive modality used to make accurate estimates of the infarcted myocardium *in vivo*. In addition, cMRI provides the ability to obtain anatomic and functional data, such as LV mass, volumes, cardiac perfusion, and regional and global myocardial functions [16-18].

The aim of this study was to combine *in vivo* cMRI and multiple postmortem techniques to establish an imaging-based theragnostic platform for evaluating rabbit models of acute myocardial infarction (AMI).

Methods

CBD and dye preparation

Cannabidiol (Tocris Bioscience, Missouri, USA) was formulated in DMSO:PEG:distilled water (1:8:10) and administered intravenously (IV) at 100 µg/kg. Control animals received the same volume of vehicle. We prepared two dyes, as described previously [19, 20]. Briefly, 1% Evans blue (EB, Sigma-Aldrich, USA) solution was injected IV at 0.5 ml/kg to specifically stain the MI-core *in vivo* [20]. In addition, the novel, custom-made dye, called red iodized oil (RIO), was prepared by diluting 20 mg oil-red-O dye (Sigma-Aldrich, USA) in 100 ml of Lipiodol ultra fluid (Guerbet, France); this was used to determine the AAR *ex vivo* [19].

Animal model of reperfused AMI

This experiment was conducted in compliance with the institutional regulations for use and care of laboratory animals (Ethische Commissie Dierproeven, KU Leuven, Belgium).

Rabbit models of AMI were prepared according to a previously introduced method [16]. Briefly, after sedation, tracheal intubation, and mechanical ventilation, rabbits received IV pentobarbital (Nembutal, Sanofi Sante Animale, Brussels, Belgium) at 40 mg/kg/h to maintain anesthesia for an open-chest operation. The left circumflex coronary artery (LCx) branch was

exposed after opening the pericardium. A 1/2 circle, triangular needle (Sutura, Inc. Fountain Valley, CA, USA) with two eyes at the end was used to place 2 silk sutures underneath the LCx. These sutures were used to make a removable coronary ligation *in vivo* and a precise re-ligation *ex vivo*. The thicker 2-0 silk suture was used for the LCx ligation and reperfusion, and the thinner 5-0 suture was saved for a later LCx re-occlusion to facilitate multifunctional staining [20]. The MI was induced by tying the thicker suture with a single detachable knot for 90 min; then, the LCx was reperfused by pulling the exteriorized suture loose in closed-chest conditions, as previously detailed [16]. An ECG was performed (Accusync® 71, Milford, Connecticut, USA) to confirm the AMI. All animals were allowed to recover, and life was sustained until the cMRI scan.

Experimental protocol

As illustrated in Figure 1B, 20 rabbits that underwent the experimental AMI-reperfusion protocol were randomly assigned into either the CBD treatment group (n=10) or the vehicle control group (n=10). Two IV injections of CBD at 100 µg/kg, or equal volumes of vehicle, were administered at 10 min before the LCx occlusion and at 10 min before the reperfusion. After 20 h, the EB solution was injected IV for postmortem visualization of the AMI-core, and 10 h later (30 h post-AMI), all animals were subjected to cMRI. They were then sacrificed, and blood was collected for a cTnI assessment. The heart was excised for *ex vivo* cMRI, RIO staining, and digital radiography (DR). The cardiac tissues were processed for histomorphology.

***In vivo* cMRI**

Rabbits were gas-anesthetized with 2% isoflurane in a mixture of 20% oxygen and 80% room air and placed in the supine position in a plastic holder. With an 8-channel phased array knee coil, the rabbits were imaged with a 3.0T clinical MRI scanner (Trio, Siemens, Erlangen, Germany) with a maximum gradient of 45 mT/m. The cMRI was triggered by both the ECG and respiration with a small animal monitoring and gating system (SA Instruments, Inc. Stony Brook, New York). Six to 10 consecutive, short-axial slices of the left ventricle (LV) were acquired with a slice thickness of 3.0 mm without gaps for all cMRI sequences. Turbo spin echo T1WI and T2WI sequences were applied to assess cardiac morphology and edema with the following parameters: TR=621/750ms; TE=15/74 ms; FOV=240×195 mm²; FA=180°; and in-plane resolution=0.9×0.9 mm². The cine-MR images were acquired in the short-axis, vertical long-axis, and horizontal long-axis planes for displaying cardiac contractions. Each cine-MRI consisted of 25 frames, spaced equally across the cardiac cycle, with a 2.5 min acquisition time. The scan parameters were: TR=357 ms, TE=1.6 ms, FOV=240×195, spatial resolution=1.2×0.9 mm^2, and flip-angle=60º. After an IV bolus injection of Gd-DOTA (Dotarem®, Guerbet, France) at 0.2 mmol/kg, three contiguous short-axis first-pass, perfusion-weighted imaging (PWI) were acquired with a segmented turbo-FLASH sequence of 80 dynamic acquisitions with the following parameters: TR/TE=241.96/1.95 ms; FOV=240×180 mm²; TI=150 ms; flip angle=15°; matrix=128×90 mm²; and in-plane resolution=1.88×1.98 mm². After 5 and 20 min, the images were acquired for early enhancement (EE) and delayed enhancement (DE) cMRIs [21] with a 3D segmented k-space

inversion recovery turbo-FLASH sequence, with the following parameters: TR=396 ms; TE=1.54 ms; TI=360 ms; FOV=240×180 mm²; FA=15°; and in-plane resolution=1.1×0.8 mm².

Postmortem procedures

Cardiac necrosis markers, cardiac Troponin I

After the cMRI scans were completed, blood was sampled in ethylenediamine-tetraacetic acid-coated tubes (Thermo Scientific Pierce, Rockford, USA) and centrifuged (2000 ×*g* for 15 min). The plasma samples were frozen and stored at -20 °C until analysis. Plasma levels of cTnI were determined with an ELISA kit (R&D, Minneapolis, US).

Macroscopic multifunctional staining

EB and RIO dyes were applied to identify the AAR, AMI-core, and the myocardial salvage zone (SZ), simultaneously, as described previously [19, 20]. Briefly, the rabbit was treated with heparin before euthanasia; then, a lateral thoracotomy was performed, and the heart was excised; a catheter was inserted into the aorta to rinse cardiac vessels with 0.9% saline. After re-ligating the LCx branch, 4 ml RIO was infused for 15 min under a pressure of 100 mmHg with the injection pump (BD PILOT, Becton Dickinson Infusion System, France). With the RIO stain, the normal myocardium appeared brick red, but the AAR remained uncolored; the MI-core had previously been stained blue *in vivo* with the EB dye.

Ex vivo cMRI

After RIO infusion, the heart was embedded in a plastic mold with 3% agar and imaged with a

high resolution T1WI spin echo sequence (TR/TE=2300/4.12; FOV=512×464 mm²; FA=10°; slice thickness=0.5 mm, and in-plane resolution=0.5×0.5 mm²).

Digital radiography

The heart was cut to produce 6-8 serial, 3.0-mm slices to match the short axis cMRIs. The cardiac slices were exposed at 25 KV/18 mA with a DR mammographic unit (Embrace; Agfa-Gevaert, Belgium).

Tissue processing, histology, and immunohistochemical staining

Heart sections were fixed with 10% formalin for 24 h. Then, the sections were paraffin-embedded, cut into 5-μm slices, and whole-mounted on a standard glass slide. The mounted slices were stained with hematoxylin-eosin (HE) and an antiserum (polyclonal rabbit anti-human; Dako A0398) specific for neutrophil myeloperoxidase (MPO) [22]. To study apoptosis, slices were stained with a terminal deoxynucleotidyl transferase biotin dUTP nick end labeling (TUNEL) kit (Roche Diagnostics, Vilvoorde, Belgium) [22]. Photomicrographs (Axioskop; Zeiss, Oberkochen, Germany, ×100-400) were interpreted by cross-referencing the cMRI with the corresponding macroscopic multifunctional staining results.

Imaging analyses

The cMRI and postmortem-stained images were analyzed with an off-line workstation equipped with dedicated software (SyngoMR A30, Siemens) and software Image J 1.38× with the planimetry method [16]. Analyses were performed by three observers blinded to the study protocol.

Myocardial edema, microvascular obstruction, and AMI in vivo

Myocardial edema and the AMI-core were defined as at least 10 adjacent pixels with signal intensities (SIs) greater than 2 SDs above the SI of the remote myocardium on a T2WI, or with SIs greater than 5 SDs above the SI of the remote myocardium on a 20-min DE-cMRI [23]. Microvascular obstruction (MVO) was defined on the 5-min EE-cMRI as a dark, subendocardial zone inside the enhanced region [18]. Regions of myocardial edema, the AMI-core, and MVO were manually traced, and their volumes were calculated by multiplying the diseased areas by the slice thickness.

LV function

Quantifications of global and regional LV functions were performed by assessing short-axis cine cMRIs with dedicated software (Argus Siemens). The endocardial and epicardial borders were manually traced in the end-diastolic and end-systolic short-axis cine images with papillary muscles included. We measured the LV end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), ejection fraction (EF), cardiac output (CO), and cardiac mass according to standard methods [16, 21]. Regional LV function was measured by wall thickening from the end diastolic phase (Fig 2A, A') to the end systolic phase (Fig 2B, B') in six clockwise sectors on short-axis cine images, excluding apical and basal slices (Fig 2) [16].

Myocardial perfusion in vivo

The first-pass PWI was used to evaluate myocardial perfusion. On each of the perfusion images, regions of interest in the LV cavity, perfusion-defective regions, and remote normal

myocardium were delineated at the first time point, then propagated with epi- and endocardial borders, throughout the imaging sequence, with manual adjustments to compensate for overall heart motion. We plotted SI-time curves to obtain relative myocardial perfusion values, including the time-to-the-peak (TTP) enhancement, the peak intensity value (PIV), the initial absolute maximal upslope (ISabs), and the area under the curve (AUC) of the contrast injection. All the PWI analyses were implemented in-house in MATLAB® (The MathWorks, Inc., Novi, MI). The pre-injection baseline SI was subtracted from the SI-time curve, and subsequently, the curves were normalized to the pre-injection baseline to correct signal reception.

Measurement of perfusion density rate

The PDR was calculated with the equation:

$$PDR = (GV_{\text{region of interest}} - GV_{\text{blank myocardium density}}) / GV_{\text{background}} \times 100\%,$$

where the PDR is the increased signal density that could be purely attributed to RIO dye perfusion; the $GV_{\text{region of interest}}$ refers to the mean gray value within the selected area; the $GV_{\text{blank myocardium density}}$ refers to the averaged gray value from the blank myocardium without the RIO dye infusion; and $GV_{\text{background}}$ represents the mean gray value from the background signal noise from the equipment [19]. The PDRs of the AAR and the normal myocardium were compared to determine relative coronary perfusion levels.

Macroscopic planimetry for standard reference

For planimetric evaluations of the different treatments, the AMI-core was defined as the EB-stained region in the heart sections. The size of the AMI-core was expressed as a

percentage (%) of the whole LV volume. The AAR was defined as the non-opaque region on the DR and the regions that were not stained with RIO in the heart sections. The AAR was expressed as a percentage of the LV. To facilitate comparisons between the different treatments, we measured the AAR/LV(%) and the AMI-core/AAR(%) with the multifunctional staining method [20]. The SZ(%) was derived as follows:

SZ% = AAR% – MI-core%.

The MSI was defined as follows: MSI = SZ% / AAR%.

Microscopic analysis

Microscopic analyses were performed by a pathologist blinded to the experimental details. On sections stained with HE and MPO, Image J software was used to semi-quantify hemorrhages and the number of infiltrating neutrophils. The scoring system used a scale of 0 to 3, where 0 was an absent reaction, 0.5 was a minimal reaction, 1 was a mild reaction observed only at high-power magnification, 2 was a moderate reaction observed at low power, and 3 was a severe reaction [22].

We also quantified the percentages of apoptotic cells that stained positive with TUNEL (brown stain) in the total area of the SZ. For quantification, heart sections were visualized with an Axiovert 200 M microscope (Carl Zeiss Inc, Gottingen, Germany), images were captured with a Zeiss Axiocam digital camera, and analyses were performed with AxioVision 4.7 software. The number of TUNEL-positive cells was counted in 10 high-power fields, randomly selected in the SZ. Apoptotic rate was expressed as the percentage of TUNEL-positive nuclei divided

by the total number of nuclei.

Statistics

Numerical data were expressed as the mean±standard deviation. The statistical analyses were performed with Analyse-it® 2.14 for Excel, GraphPad Prism® 5.0. We used ANOVA and Bonferroni's posthoc tests to analyze differences between groups. When data were not normally distributed, we performed Kruskal-Wallis nonparametric statistics, and differences were identified with Mann-Whitney or Wilcoxon tests. A linear regression was used to determine correlations between *in vivo* and *ex vivo* evaluations of the therapy. All tests were two tailed. A p-value <0.05 was considered statistically significant.

Results

General aspects

All rabbits survived the systemic anesthesia, open-chest surgery, and cMRI. None exhibited observable toxic or adverse effects after intravenous administration of either the CBD or the EB dye. In all cases, the presence of AMI was indicated *in vivo* with characteristic ECG profiles and cMRIs; AMI was confirmed postmortem with the multifunctional staining and histopathology.

Cardiac necrosis markers in blood

The CBD treatment group had significantly lower peak cTnI (ng/ml) values compared to the control group (3.99±1.95 vs. 6.39±2.93; p=0.02).

LV function

The two groups were not significantly different in quantitative parameters of global LV function. However, the CBD group tended to show increases compared to controls, respectively, in the SV (2.21±0.53 vs. 1.78±0.39 ml), CO (0.27±0.03 vs. 0.20±0.07 l/min), and EF (45.36±8.72 vs. 38.93±4.65%).

On cine cMRIs (Fig. 2A, A', B, B'), an analysis of regional wall thickening showed that the wall motions were impaired on the anterior, lateral, and inferior LV walls compared to the normal LV walls in both groups. However, CBD significantly improved the thickening of the lateral LV wall (CBD: 34.83±8.15% vs. controls: 25.35±9.89%; $p<0.05$; Fig 2C).

Regional perfusion *in vivo* and perfusion density rate *ex vivo*

The first-pass PWI showed a perfusion defect in the LCx region in both groups (Fig 3A, A'). The SI-time curves revealed dynamic SI changes within the left ventricular cavity, normal myocardium, and AAR (Fig 3B, B'). The two groups showed significant differences in the PWI parameters, including the PIV and the AUCs measured at 15, 30, and 45 min ($AUC_{15'}$, $AUC_{30'}$, and $AUC_{45'}$; Table 1). These differences indicated that CBD significantly increased the relative blood flow in the perfusion-defective region (the AAR; $p<0.05$).

The PDRs in the normal myocardium and in the perfusion-deficient region (AAR) were examined with DR (Fig 3C, C'). The PDRs were 347.6±33.1% and 30.2±13%, respectively, in the control group, compared to 377.2±37.2% and 52.2±14.3%, respectively, in the CBD group. The PDR in the AAR was about 1.7 times (52.2/30.2) higher with CBD-treatment than in

control conditions. Thus, CBD appeared to increase blood perfusion in the AAR, consistent with the *in vivo* PWI findings.

Effect of CBD on edema and MVO

The T2WI results showed a homogeneous, high SI over the culprit vascular regions in both groups. Although the two groups showed no significant difference in edema volume (p=0.445), the CBD group showed slightly less edema than the controls (43.21±10.81 vs. 46.84±13.44%). EE-cMRI showed 15 rabbits with MVO (8 controls, 7 CBD-treated) and 5 without MVO. In both groups, relative to small AMIs, the large AMIs tended to be associated with regions of "no-reflow" or MVOs. The CBD group showed significantly smaller regions of MVO compared to the control group (7.25±2.3% vs. 14.91±4.17%; p=0.01, Fig 4A-C, A'-C', E). This finding was supported by the micro thromboembolisms observed in histological analyses (Fig 4D, D').

CBD effects on the AAR/LV(%), AMI-core/AAR(%), and MSI

Transmural hyper-enhanced zones on the DE-cMRIs indicated necrotic AMI-cores (Fig 5A, A'), similar to those found on *ex vivo* DE-cMRIs (Fig 5B, B') and on EB-stained specimens (Fig 5D, D'). The AMI-cores appeared somewhat smaller than the AARs, defined by non-opacified regions on the DR (Fig 5C, C') and by regions not stained with RIO in tissue sections (Fig 5D, D'). In the control group, the mean AMI-core/LV (35.35±12.64%) measured on *in vivo* DE-cMRIs correlated well with the *ex vivo* DE-cMRI findings (34.77±10.38%; r^2=0.95) and the EB-stain findings (33.45±12.21%; r^2=0.90). In the CBD group, the mean

AMI-core/LV (30.45±11.34%) measured on *in vivo* DE-cMRIs also correlated well with the *ex vivo* DE-cMRI findings (29.89±13.71%; r^2=0.94) and with the EB-stain findings (27.45±10.66%; r^2=0.92). There were no significant differences between the two groups in the AAR/LVs (50.89±15.94% vs. 48.49±16.89%; p=0.85; Fig 6A). However, in the CBD group, the AMI-core/AAR was lower than controls (60.51±12.94% vs. 72.66±12.64%, respectively; p<0.05; Fig 6B) and the MSI was higher than controls (0.39±0.12% vs. 0.27±0.13%, respectively; p<0.05; Fig 6C). Thus, CBD administration significantly reduced the AMI size and increased the MSI compared to the vehicle control.

Histopathological findings

Macroscopy clearly revealed the hemorrhagic AMI-core in contrast to the lightly-stained surrounding myocardium (Fig 5E, E'). Topographically, these results were similar to those observed with multifunctional staining (Fig 5D, D'). Microscopy revealed more prominent hemorrhages in the AMIs of controls compared to those in the CBD group (Fig 7A, A'). The hemorrhage score was lower in CBD-treated than in control rabbits (0.98±0.39 vs. 2.32±0.96; p<0.05). In all cases, neutrophils were stained with MPO. The density of MPO-stained granules was significantly higher in the control group than in the CBD group (92% vs. 34%, p<0.05), which indicated greater neutrophil infiltration in control rabbits (Fig 7B, B'). The cell death rate, measured by the number of TUNEL-positive nuclei, was significantly lower in the CBD group compared to control rabbits (18% vs. 48%, p<0.05; Fig 7C, C').

Discussion

Our results showed that two injections of CBD (100 μg/kg), delivered 10 min before LCx occlusion and reperfusion, exerted cardioprotective effects on acute ischemia-reperfusion injury. The main outcomes of this study were: 1) CBD induced no detectable undesired toxic or psychological effects ; 2) CBD decreased the blood cTnI levels; 3) CBD increased blood flow in the AAR, and thus, preserved some cardiac functions; 4) CBD reduced the AMI-core/AAR% and increased the MSI; 5) CBD provided protection from reperfusion injury, and this protection was associated with reductions in infiltrating neutrophils and MPO activity; and 6) we successfully evaluated the therapeutic effects of CBD on an occlusion/reperfusion rabbit model, based on our previously established technical platform.

Ischemia-reperfusion injury is a major problem after AMI. The treatment options are limited, but they are evolving with research in both *in vitro* and *in vivo* models [1, 24]. CBD is a non-psychotropic constituent of the *Cannabis sativa* (marijuana) plant, which was reported to exert antioxidant and anti-inflammatory effects, both *in vitro* and in various preclinical models of neurodegenerative and inflammatory disorders [8-12]. The effects of CBD are independent of those found in conventional approaches that targeted CB1 and CB2 receptors [25]. Both prolonged and transient CBD administrations have demonstrated cardioprotective effects, but only in rat models [2, 7]. The present study was the first to test CBD in medium-sized animals (rabbits) with a myocardial occlusion/reperfusion insult. We showed that two consecutive CBD injections provided acute cardioprotection by increasing the regional blood flow,

improving the wall thickness, and attenuating the AMI size.

Our dosing protocol was similar to that used previously by Walsh *et al.*, [7] except that we doubled each CBD dose from 50 to 100 μg/kg; this dose was deemed clinically-relevant and its safety was supported by the fact that rabbits tolerated this higher dose well without observable side effects. In a review, Bergamaschi *et al.* [9] found that chronic CBD administrations and high doses up to 1500 mg/day were well-tolerated in humans.

Cardiac troponins regulate contractile function in striated muscle. Their presence in the blood indicates cardiac damage [26, 27]. Indeed, cTnI is uniquely expressed in the heart, and it provides a specific biomarker of AMI [27]. The diagnostic value of cTnI has been rarely studied in animals with AMI that received CBD treatment. In our study, the blood cTnI level significantly decreased after CBD treatment, which indicated the cardioprotective effect of CBD. Furthermore, we observed that neutrophil migration and MPO activity were significantly reduced in CBD-treated rabbits. These findings may be attributable to a protective effect of CBD on the microvasculature. MPO is a good surrogate for tissue neutrophil activity, which occurs predominantly under pathological conditions [28]. MPO-associated tissue damage was probably inhibited by CBD, which contributed to a reduction in myocardial inflammation.

The cMRI noninvasively provides abundant morphological information about myocardial edema, and it reveals the location, transmurality, and size of AMIs, MVOs, and intramyocardial hemorrhages. Moreover, the cMRI facilitates accurate assessments of global and regional LV functions. Currently, the cMRI represents the most powerful phenotyping tool for comprehensive evaluation of post-infarction remodeling [29]. In this study, the cMRI was

applied mainly to evaluate therapeutic effects on cardiac function and perfusion, because the morphology could be quantified with standard postmortem techniques. Although CBD did not significantly improve global LV function, it significantly increased the thickness of the lateral wall in the dominant region affected by the LCx. The first-pass PWI offered dynamic information about the passage of the Gd-contrast agent through the entire heart. This technique was used to assess blood perfusion in the ischemic versus the normal myocardium, which formed the basis of the perfusion reserve indices [17]. More recently, the perfusion reserve has been estimated from the AUC of the myocardial SI over time, up to the observation of the first pass peak in the blood. This AUC served as an indicator of myocardial blood flow, and it was previously validated against microsphere measurements [17, 30]. We performed semi-quantitative measurements of the parameters derived from myocardial SI-curves, and we found that CBD therapy significantly increased the PIV and AUC within the AAR, from baseline to 15, 30, and 45 min. Therefore, CBD seemed to improve microvascular flow, which was consistent with our observations of reduced MVO and increased regional wall thickening.

The mechanisms of action for CBD remain obscure. CBD reportedly reduced brain damage and improved functional recovery after acute hypoxia-ischemia in newborn pigs [31]. Other groups have indicated that CBD had anti-inflammatory effects in a murine model of acute lung injury, and that the effect was associated with an increased supply of extracellular adenosine, which stimulated signaling through adenosine A2A receptors [28]. Moreover, several studies have shown that CBD was beneficial in preventing ischemia-reperfusion damage. In the liver, ischemia/reperfusion caused significant elevations in alanine aminotransferase, hepatic

malondialdehyde, tumor necrosis factor-alpha, and nitric oxide levels. CBD counteracted those effects by attenuating inflammatory signaling and responses, oxidative and nitrative stress, and cell death [25, 32], and by significantly reducing the expression of inducible nitric oxide synthase [32]. Liu et al showed that increasing the nitric oxide levels could improve microvascular flow and decrease AMI size after myocardial ischemia and reperfusion in pigs [22].

CBD was also found to bind to PPARγ and stimulate the differentiation of 3T3-L1,1 fibroblasts into adipocytes [14]. Ligand-activated PPARγ increased anti-inflammatory cytokines, inhibited inducible nitric oxide synthase expression [33], and decreased the inflammatory response in cardiovascular cells, particularly endothelial cells [34]. Furthermore, CBD decreased monocyte adhesion and transendothelial migration. These properties indicated that CBD may provide significant therapeutic benefits to patients with diabetic complications and atherosclerosis [35].

In 2007, Durst and colleagues showed that *in vivo* treatment with CBD significantly reduced the AMI size in rats, and this was associated with a reduction in infiltrating leucocytes and circulating interleukin (IL)-6 [2]. In 2010, Walsh et al. subsequently showed that a single IV dose of CBD at 50 mg/kg, given 10 min pre-ischemia or 10 min pre-reperfusion, could significantly reduce the infarct size [7]. This effect was associated with a reduction in ventricular ectopic beats, which suggested that CBD might also act as an anti-arrhythmic agent. Also in 2010, Rajesh et al. showed that, after 11-weeks of *in vivo* CBD treatment (20 mg/kg, i.p.), diabetic mice showed significant reductions in cardiac dysfunction. This effect was associated with reduced myocardial inflammation, oxidative stress, nitrative stress, and fibrosis, mediated by reduced nuclear factor-κB activation, reduced mitogen-activated protein kinase activation, and reduced

expression of adhesion molecules and tumor necrosis factor [15]. In the present study, although we measured fewer biochemical markers, we confirmed the cardioprotective effects of CBD in a rabbit ischemic-reperfusion model. The role of CBD may be related to the above-mentioned mechanisms, but they need to be verified further.

Methodologically, the experimental techniques introduced and applied in this study provided specific staining of the AMI-core with EB dye and clear visualization of the AAR. The DR showed a filling defect, and ROI staining indicated the damaged, non-stained regions on heart sections; moreover, the whitish areas indicated salvageable myocardium on heart sections.

This study had some limitations. First, our attempts to image the contracting rabbit heart with a clinical MR scanner proved technically challenging; the artifacts from respiratory movements and heartbeats could not be completely eliminated. Second, we did not attempt to evaluate AAR and MSI with *in vivo* cMRI scans, which are thought to be clinically crucial. Third, the CBD dosage protocol requires optimization to maximize the therapeutic effects of CBD.

Conclusion

In this study, we demonstrated that cMRI could be used to monitor, noninvasively, the beneficial effects of the newly-identified cardioprotective agent, CBD, on a rabbit model of AMI. In addition, our multifunctional staining method demonstrated that CBD treatment reduced the size of ischemic injury in the myocardium.

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Conflict of interest

None.

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Figures legends

Figure 1. Introduction to cannabidiol (CBD) and the study protocol. A: Introduction to the *Cannabis sativa* plant and the chemical structure of CBD. B: A flowchart of the study protocol.

Figure 2. Comparison of LV wall thickening between control and CBD groups.

LV end diastolic (A, A') and systolic (B, B') cine short-axis images from CBD-treated and control groups. We segmented the LV wall into six equal sectors to evaluate wall thickening. The blue and red dashed-lines correspond to control and CBD groups on the histogram. Sectors 1-3 show decreased wall thicknesses in both groups, but CBD significantly increased the wall thickness in sector 2. Sector 1: left anterior wall; Sector 2: lateral wall; Sector 3: left inferior wall; Sector 4: right inferior wall; Sector 5: septal wall; and Sector 6: right anterior wall. *$p<0.05$ indicates a significant difference between groups

Figure 3. *In vivo* myocardial first-pass perfusion (PWI) and *ex vivo* perfusion digital radiography (DR) for control and CBD groups. (A, A') The PWI showed a perfusion deficit after the LCx occlusion and reperfusion insult, which was larger in (A) the control group than in (A') the CBD group. (B and B') SI-time curves indicate slower and lower contrast enhancement in the AAR compared to that in the normal myocardium (NM) and in the ventricular cavity (VC); perfusion was slightly increased in the CBD group. (C and C') The DRs showed that the AAR of the control group was larger than that of the CBD group. The AAR detected with *in vivo* PWI was a good match to that detected with the *ex vivo*

DR in both groups.

Figure 4. Evaluation of microvascular obstruction (MVO) with cMRI and histomorphology.

(A, B, C) The MVOs from base to apex in the control group were apparently larger than (A', B', C') those in the CBD group. (D, D') HE-stained micrographs showed severe MVO with more thromboembolic material in the microcirculation of (D) control animals compared to (D') CBD-treated animals. (E) The histograms of cMRI data indicate that CBD significantly decreased MVO.

Figure 5. Comparison of large hemorrhagic AMIs between control (upper row) and CBD (lower row) groups. (A, A') On DE-cMRIs, transmural AMIs appeared hyperenhanced, which corresponded well to (B, B') *ex vivo* DE-cMRI findings; (C, C') with DR, AARs are shown as perfusion deficits; (D, D') with RIO-staining, AARs appear as negative regions. The EB-stained, dark blue MI-cores within the RIO-negative AARs became apparently smaller after CBD therapy; (E, E') HE-stained photographs confirmed the CBD effect on AARs..

Figure 6. Effect of CBD on the AAR/LV (%), AMI-core/AAR (%), and myocardial salvage index (AAR–AMI-core)/AAR), defined by multifunctional staining. (A) There was no significant difference between the two groups in the AAR/LV (%). (B) CBD significantly decreased the AMI size, based on the AMI-core/AAR (%), and (C) CBD increased the MSI. *p <0.05 significant difference between groups

Figure 7. Histochemical staining of myocardial sections treated with or without CBD. (A, A') HE staining shows severe hemorrhage in the infarct zone of (A) a control heart section, but not (A') in a CBD-treated heart section. (B, B') Myeloperoxidase (MPO) immunostaining in the AAR indicates abundant neutrophil infiltration in (B) the control heart, but less severe in (B') the CBD-treated heart. (C, C') TUNEL staining indicates numerous apoptotic myocytes in the AAR of (C) control hearts, but much less in (C') the CBD-treated hearts. Original magnifications: (A, A') ×100, (B, B') ×200, and (C, C') ×400

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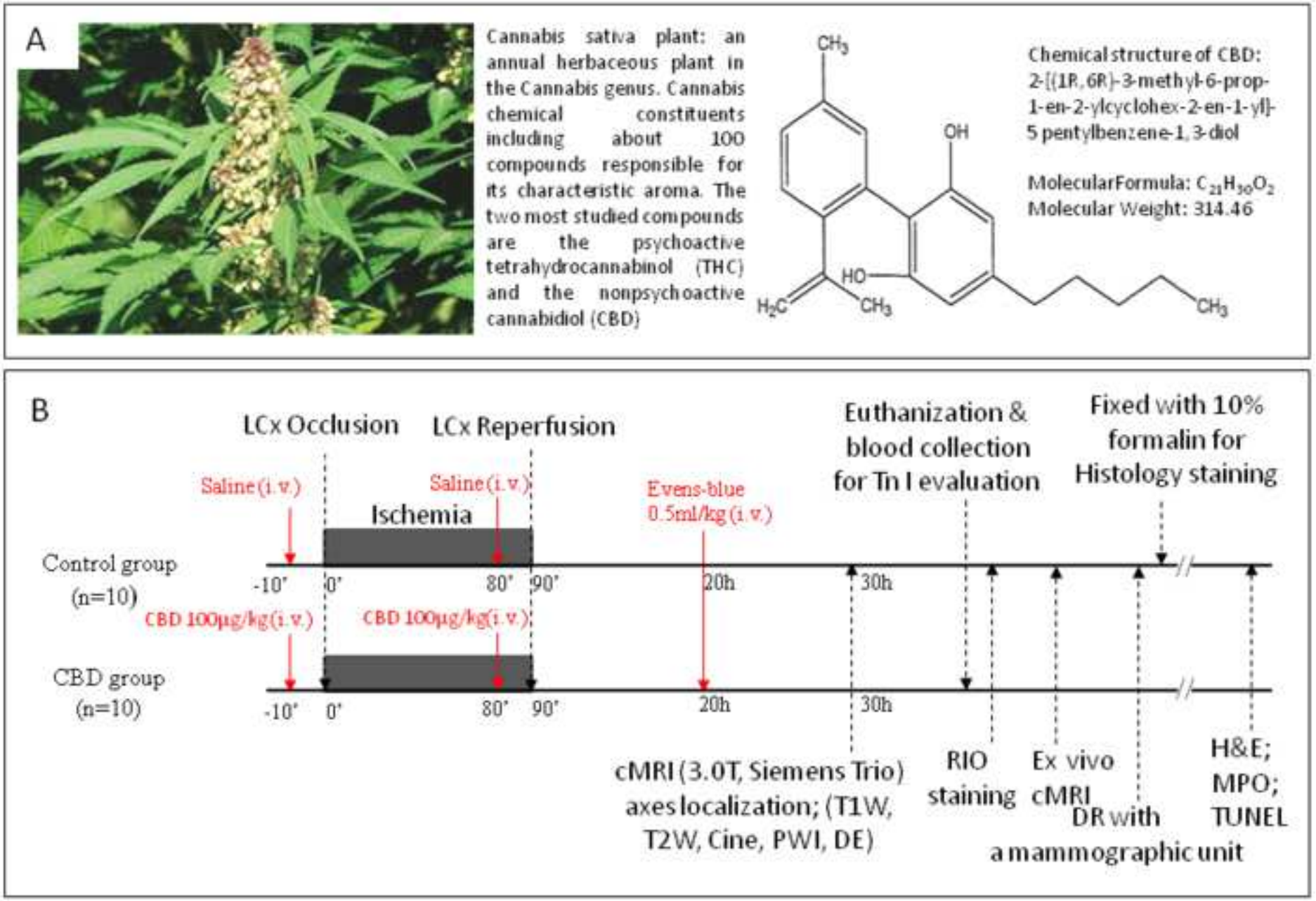

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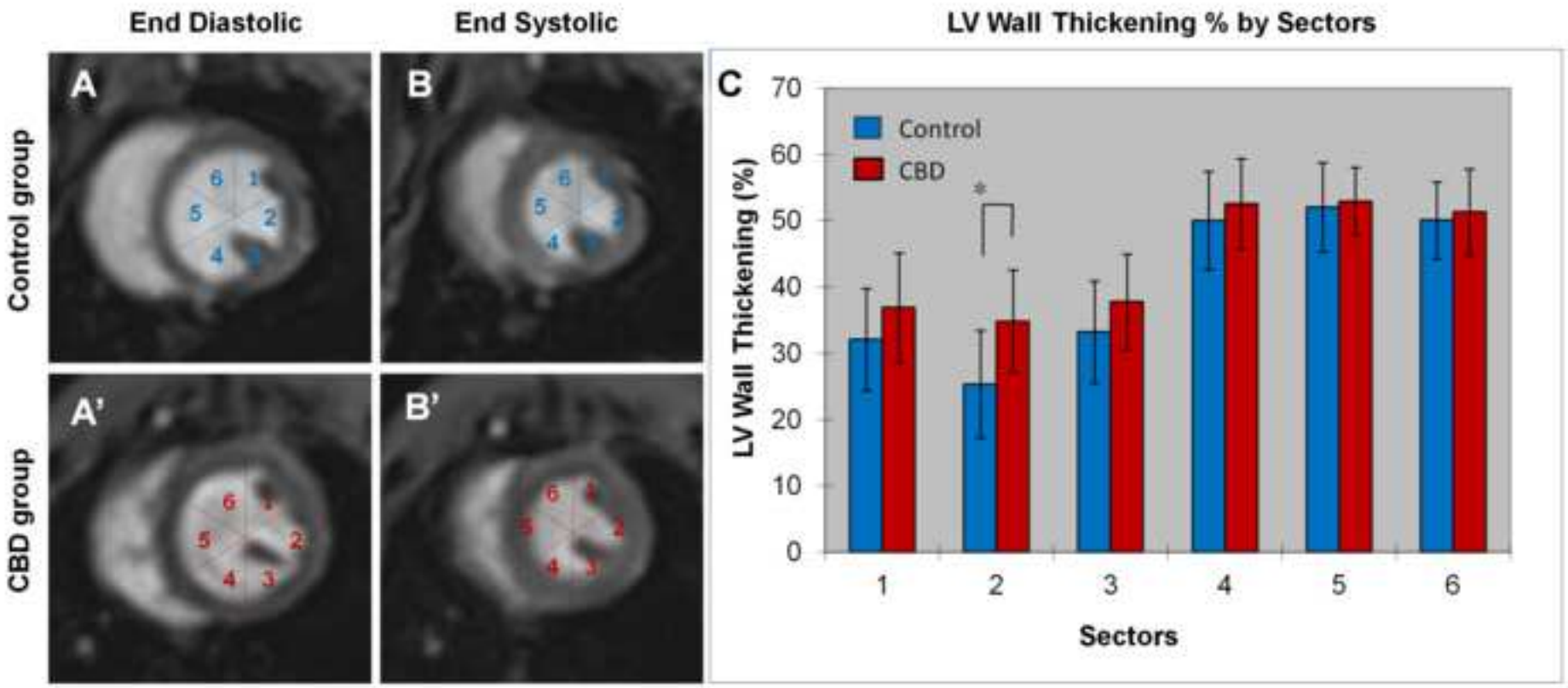

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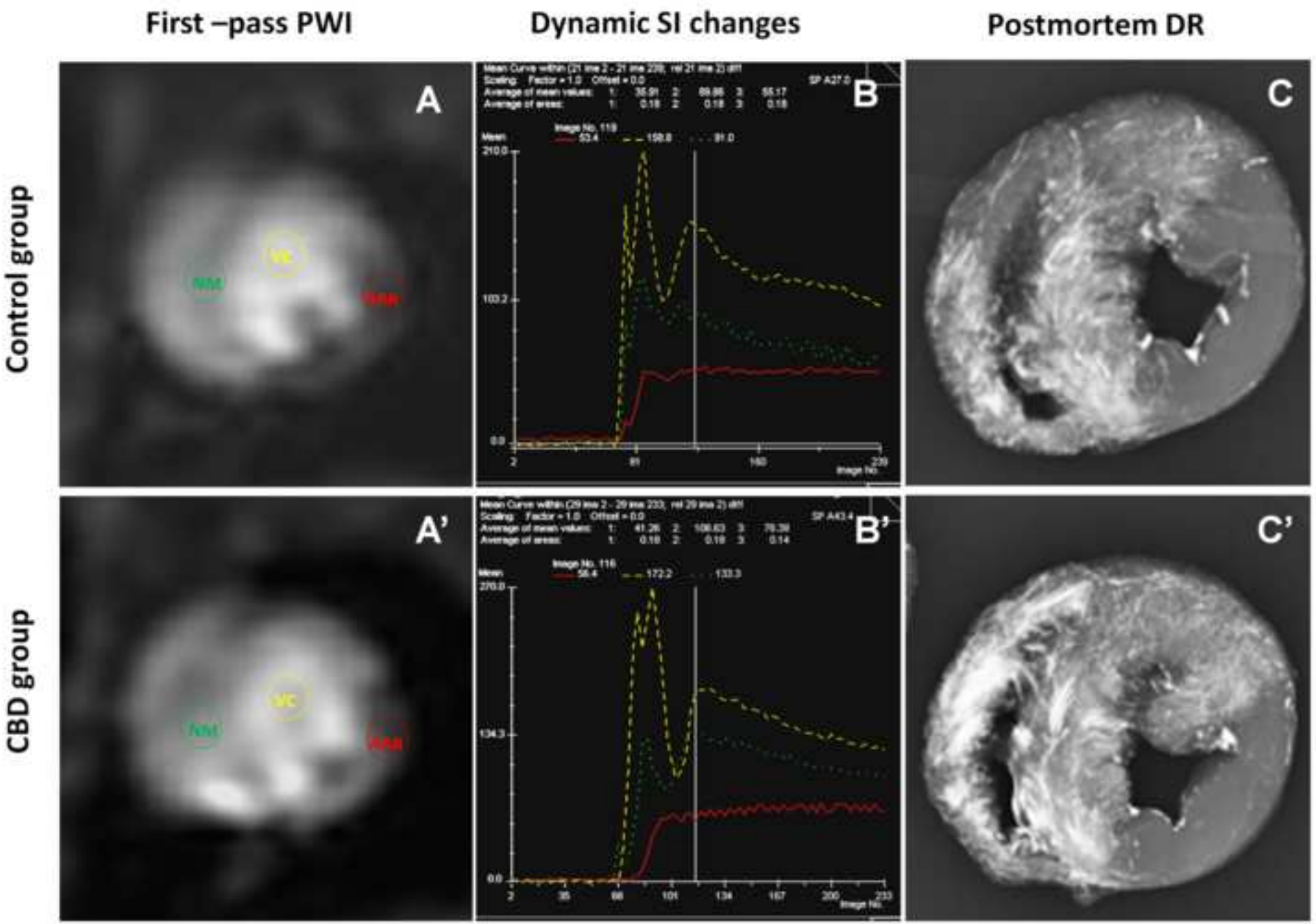

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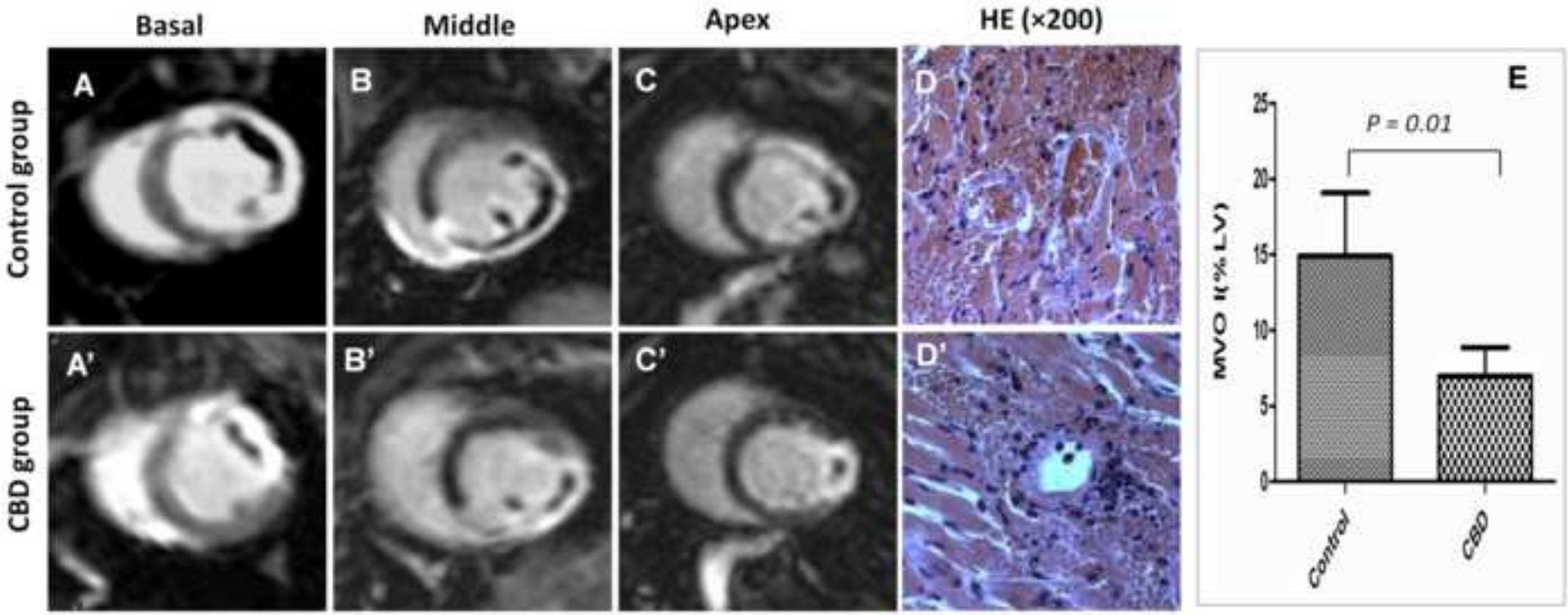

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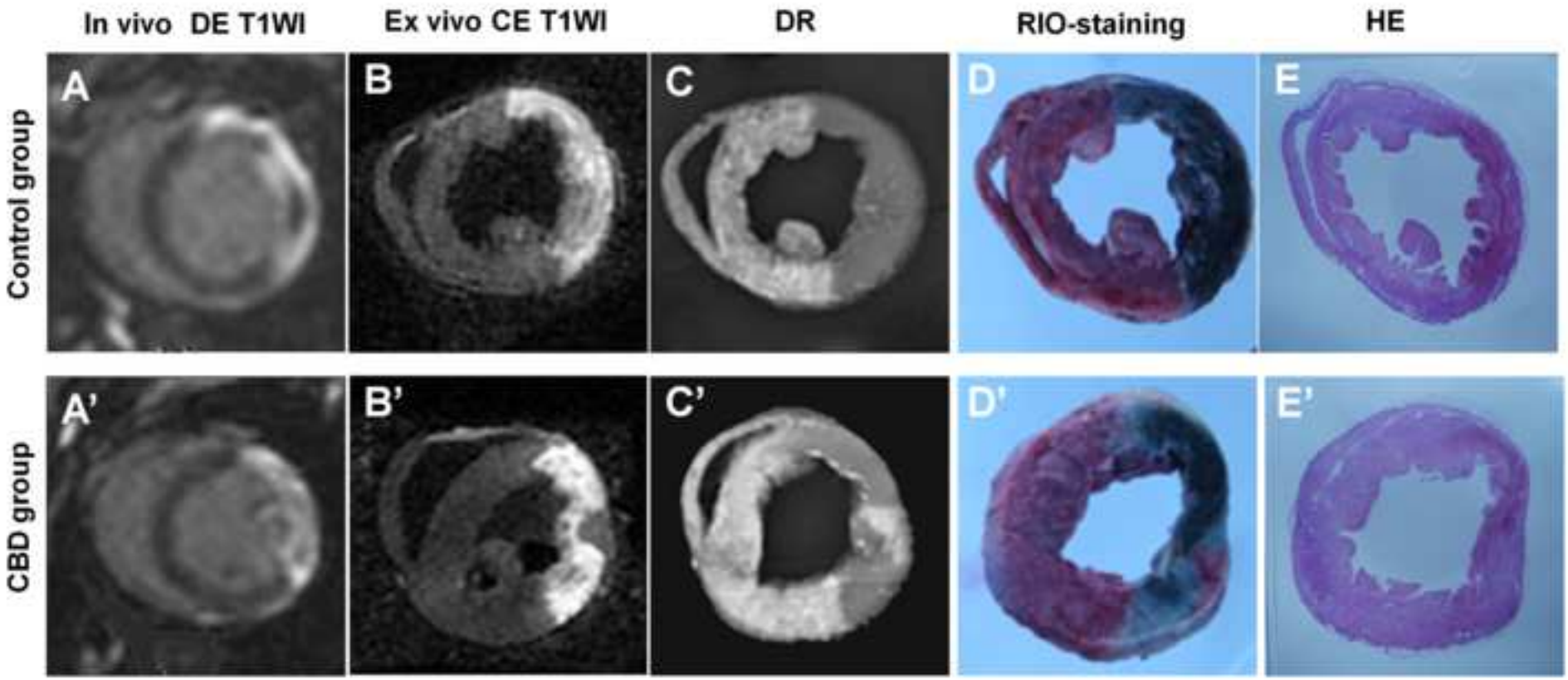

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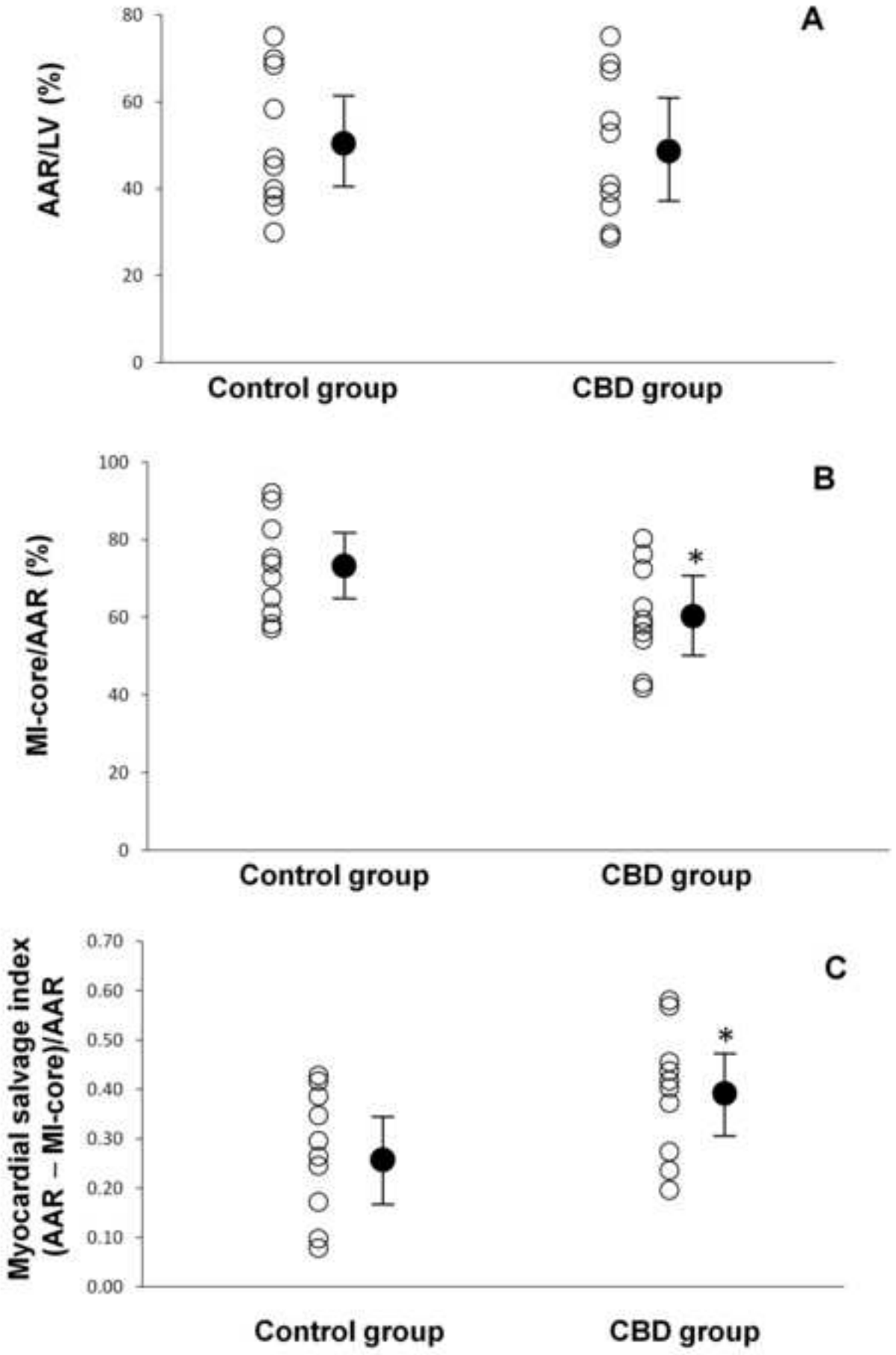

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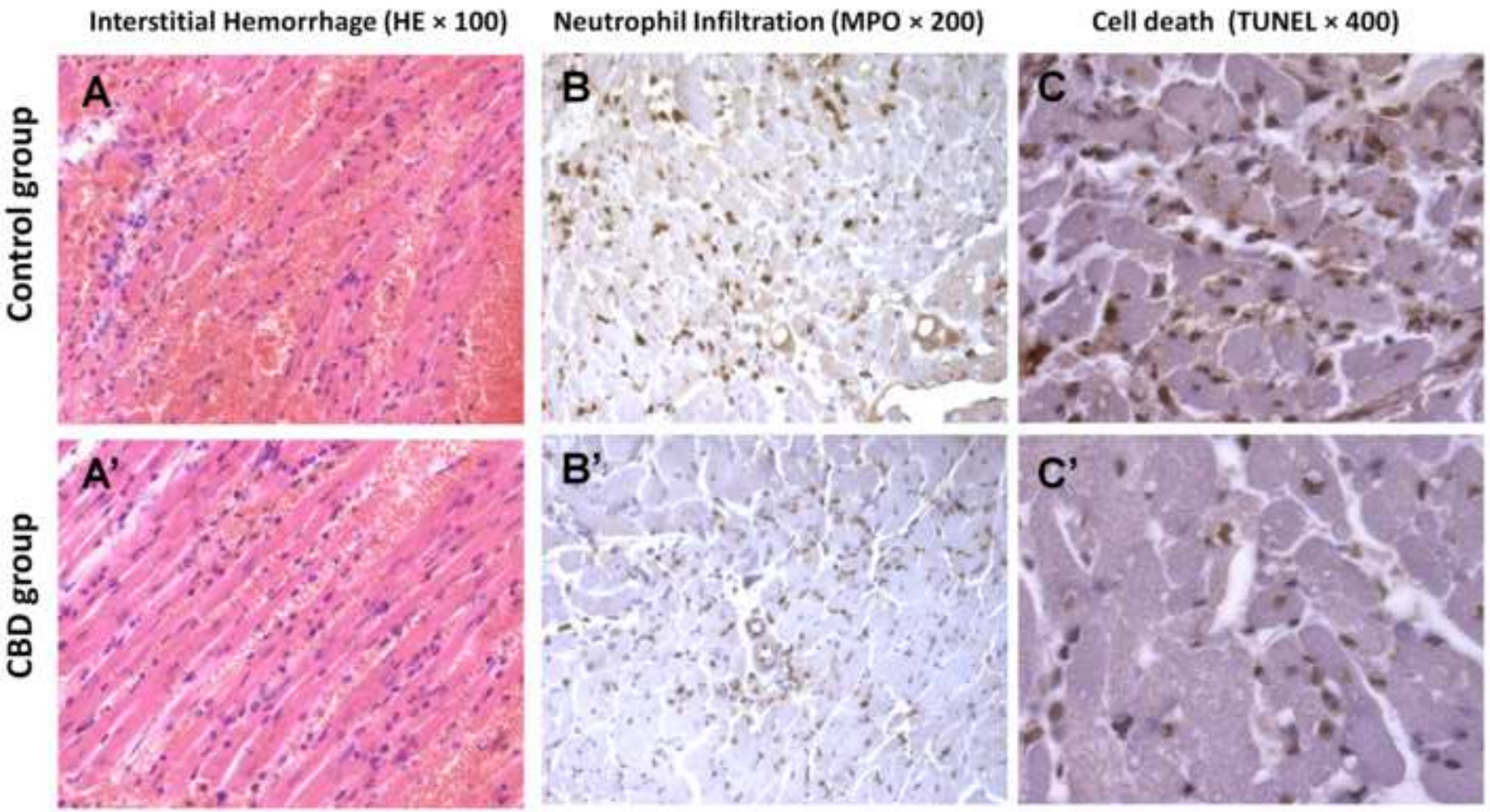

Table

Table 1. Evaluation on myocardial perfusion after therapy.

	Ventricle cavity		Normal myocardium		Perfusion deficit (AAR)	
	Control	CBD	Control	CBD	Control	CBD
PIV: (SI)	13.14±1.67	12.75±1.69	6.42±1.03	6.52±1.21	3.66±1.82	5.22±2.21*
TTP(sec)	8.68±3.3	7.16±1.89	14.19±6,08	12.45±5.87	53.32±19.1	46.79±20,32
IS_{abs}(SI/sec)	87.72±32.11	86.13±35.06	37.86±14.49	40.85±21.78	14.73±5.68	17.81±7.73
AUC_{15}(SI*sec)	114.07±26.52	120.89±22.62	52.55±12.69	55.21±10.16	23.22±7.31	32.29±10.22*
AUC_{30}(SI*sec)	233.39±33.81	234.41±20.63	121.78±18.65	126.97±18.22	59.76±17.57	83.64±20.23*
AUC_{45}(SI*sec)	355.86±38.97	363.30±38.73	187.43±37.09	201.22±30.27	99.17±21.95	148.92±32.63*

Data are presented as mean ± SD. PIV: peak intensity value; SI: signal intensity; TTP: time to peak; ISabs: initial absolute maximal upslope; AUC_{15}, AUC_{30}, AUC_{45}: area under curves from base to 15', 30' and 45'.

*: $P<0.05$ obtained from a two-way ANOVA and Bonferroni post hoc test indicates a significant difference.